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Characterization of the ternary complexes formed in the reaction of cis-diamminedichloroplatinum (II), ethidium bromide and nucleic acids

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ABSTRACT

The purpose of this study was to characterize the ternary complexes formed in the reaction of cis-diamminedichloroplatinum (II) (cis-DDP) and nucleic acids, in the presence of the intercalating compound ethidium bromide (EtBr). In these ternary complexes, some EtBr is tightly bound to the nucleic acids. Tight binding is defined by resistance to extraction with butanol, assayed by filtration at acid pH or thin layer chromatography at basic pH. These ternary complexes are formed with double stranded but not with single stranded nucleic acids. They are not formed if cis-DDP is replaced by trans-diamminedichloroplatinum(II). The amount of tightly bound EtBr depends upon the sequence of the nucleic acid, being larger with poly (dG-dC).poly(dG-dC) than with poly(dG).poly(dC). Spectroscopic results support the hypothesis that the tight binding of the dye is due to the formation of a bidentate adduct (guanine-EtBr)cis-platin. The visible spectrum of the ternary complexes is blue-shifted as compared to that of EtBr intercalated between the base pairs of unplatinated DNA and it depends upon the conformation of the ternary complex. The fluorescence quantum yield of the ternary complexes is lower than that of free EtBr in water. Tightly bound EtBr stabilizes strongly the B form versus the Z form of the ternary complex poly(dG-dC)-Pt-EtBr and slows down the transition from the B form towards the Z form. The sequence specificity of cis-DDP binding to a DNA restriction fragment in the absence or presence of EtBr is mapped by means of the 3'→5' exonuclease activity of T4 DNA polymerase. In the absence of the dye, all the d(GpG) sites and all the d(ApG) sites but one in the sequence d(TpGpApGpC) are platinated. The d(GpA) sites are not platinated. In the presence of EtBr, some new sites are detected. These results might help to explain the synergism for drugs used in combination with cis-DDP and in the design of new chemotherapeutic agents.

INTRODUCTION

Numerous studies have been devoted to the study of the reaction of the antitumor drug cis-diamminedichloroplatinum(II) (cis-DDP) and DNA. The mechanism of cis-DDP action is not yet known but it is often thought that its antitumor activity is related to its binding to DNA (1-4) . In the in vitro reaction between cis-DDP and DNA, the two major adducts derive from intrastrand cross-links of cis-DDP on d(GpG) and d(ApG). Minor adducts derive from intrastrand cross-links of cis-DDP on two guanine residues separated by one

base and from interstrand cross-links of cis-DDP on two guanine residues (5-12).

The DNA conformation plays an important role in the binding of the drug. It has be found that the relative percentages of adducts depend on whether cis-DDP reacts with native or denatured DNA (12). In the reaction of cis-DDP and poly(dG-m^5dC).poly(dG-m^5dC) a bidentate or a monodentate adduct is formed depending on whether the polynucleotide is in the B or in the Z form (13). The presence of intercalating dyes can interfere with both the position and the mode of cis-DDP binding to DNA. By means of exonuclease III mapping experiments, it has been proposed that intercalating dyes such as ethidium bromide can modulate the local structure of DNA and thus influence the selectivity of cis-DDP binding (14-16). In a recent work we have found that incubation of double stranded nucleic acids and cis-DDP in the presence of some intercalating dyes such as ethidium bromide or proflavine leads to the formation of ternary complexes in which the dyes are tightly bound to the nucleic acids (17). Tight binding is defined by resistance to extraction with butanol, assayed by filtration at acid pH or by thin layer chromatography at basic pH. To explain this strong binding, it has been suggested that cis-DDP cross-links a guanine residue and a dye. Moreover, it has been shown by competition experiments that ethidium bromide, proflavine and also acridine (which does not form a tightly bound complex) interfere with the preferential binding of cis-DDP to $(dG)_n.(dC)_n$ sequences.

We here report some more results on the tightly bound complexes formed between natural and synthetic double stranded polynucleotides, cis-DDP and ethidium bromide. Spectroscopic studies (visible absorption, fluorescence and circular dichroism) show that the dye and platinum are in close contact. The tightly bound dye stabilizes the B form versus the Z form of the ternary complex poly(dG-dC)-Pt-EtBr and reduces the transition rate from the B form to the Z form. The sequence specificity of platinum binding in the absence and in the presence of ethidium bromide was mapped by means of the 3' → 5' exonuclease activity of T4 DNA polymerase.

MATERIALS AND METHODS

Double stranded polynucleotides, purchased from Boehringer Manheim and Pharmacia, Micrococcus luteus DNA prepared as already described (18) were treated twice with phenol and then precipitated with ethanol. Stock solutions of nucleic acids were made in 10 mM $NaClO_4$/1 mM phosphate buffer pH 7.5. Unlabelled ethidium bromide (Sigma) and [C-^{14}] ethidium bromide (0.7

GBq/mmole) (Centre de l'Energie Atomique, France) were used without any further purification. 5'-[-^{32}P] ATP (111 TBq/mmole) was obtained from Amersham. cis-DDP and trans-DDP were kindly provided by Dr J.L. Butour.

The restriction enzymes Sal1 and BamH1, alkaline phosphatase were purchased from Boehringer Manheim, T4 polynucleotide kinase from BRL, T4 DNA polymerase from Biolabs. Enzyme buffers were those recommended by the suppliers.

Ultrapure agarose was from BRL and electrophoresis-grade acrylamide and bis-acrylamide from Amersham.

Reactions of cis-DDP and nucleic acids, in the presence or absence of ethidium bromide, as well as the determination of the amount of tightly bound ethidium by filter assay at acid pH, by thin layer chromatography at basic pH or by butanol extraction, have been previously described (17). The platinum contents of the samples were determined with an atomic absorption spectrophotometer by Dr J.L. Butour.

The DNA restriction fragment (275 bp) was obtained as previously described (19) and summarized as follows : 10 µg of pBR322 was treated with Sal1 (1 h at 37°C, 1U/1 µg of DNA) to obtain linear plasmid followed by treatment with alkaline phosphatase (2x30' at 50 °C, 2x2U/100 µg of DNA). Then linear pBR322 was 5'-end labelled by treatment with T4 polynucleotide kinase (30' at 37°C 10U/10 µg of DNA) in the presence of 50 µCi of 5'-[-^{32}P] ATP. The 275-bp fragment was obtained by BamH1 treatment and was isolated by electrophoresis through a preparative 1 % agarose gel with Schleicher and Schuell membrane filters.

Platination of the restriction fragment in the absence or in presence of ethidium bromide was carried out in 10 mM $NaClO_4$/1 mM phosphate buffer pH 7.5 containing 0.7 µg of labelled 275 bp fragment in 60 µl during 20 hours at 37°C in the dark. The dye was removed by three butanol extractions, followed by two ether washes and ethanol precipitation. Then platinated samples were resuspended in deionized water and a 10-fold concentrated solution of T4 DNA polymerase buffer was added to find conditions in which this enzyme has 3' → 5' exonuclease activity. 7 units of enzyme were added to 0.25 µg of DNA and incubation was run for 40 minutes at 37°C. Then the enzyme was extracted with phenol, followed by two ether washes and ethanol precipitation. To remove platinum, incubation with 0.2 M NaCN was performed for one night at 37°C.

For sequencing samples, 150 cpm were loaded on a 0.8 mm, 8 % polyacrylamide - 7 M urea gels with TBE running buffer and electrophoresed at 70 V/cm for about 3 hours. Maxam-Gilbert base specific reactions were electrophoresed

in parallel to provide a sequence ladder (20). Gels were then fixed in 10 % acetic acid, 10 % methanol aqueous solution, applied on a gel dryier and exposed on Fuji X Ray film over night at room temperature.

Absorption and circular dichroism spectra were recorded with a Kontron Uvikon 810 spectrophotometer and with a Jobin-Yvon III dichrograph, respectively. Fluorescence measurements were performed on a Mark I spectrofluorometer.

The following abbreviations have been used : cis-DDP, cis-diamminedichloroplatinum(II) ; trans-DDP, trans-diamminedichloroplatinum (II) ; EtBr, ethidium bromide ; nucleic acid-Pt-EtBr, ternary complex obtained after incubation of cis-DDP, EtBr and nucleic acid reaction mixture and then extraction with butanol ; r_b, molar ratio per nucleotide.

RESULTS

Formation of the ternary complexes

EtBr forms reversible complex with DNA. All the bound dye can be removed by extraction with organic solvants (butanol, phenol), by filtration at acid pH and by tlc at basic pH. After reaction of cis-DDP and DNA, the platinated DNA binds EtBr and all the bound EtBr can be removed from this platinated DNA by the same assays used with unplatinated DNA. The behavior of DNA which has been reacted with cis-DDP in the presence of EtBr is different. As previously described (17), when double stranded nucleic acids were first mixed with EtBr and then cis-DDP was added, after a few hours of incubation of the reaction mixture at 37°C, some EtBr molecules, called tightly bound EtBr, could not be removed by extraction with butanol, by filtration at acid pH and by tlc at basic pH. The molar ratio r_b (tightly bound EtBr per nucleotide) depended upon the relative concentration of cis-DDP, EtBr and nucleic acids. We here report some results on the importance of the base sequence of the nucleic acid. As shown in fig 1, in all the cases, r_b increases as a function of time and then after about 20 hours, remains constant. However, the values of r_b are different, r_b poly(dG-dC) > r_b poly(dA-dC).poly(dG-dT) > r_b(DNA) > r_b (poly(dG).poly(dC). The amount of bound platinum as determined with an atomic spectrophotometer is independent of the base composition of the nucleic acid (not shown). The reaction is specific of cis-DDP. If cis-DDP is replaced by the trans isomer, no tightly bound EtBr is found (results not shown).

The structure of the nucleic acid plays an important role. As shown in fig 2, the amount of tightly bound EtBr is smaller with denatured DNA than

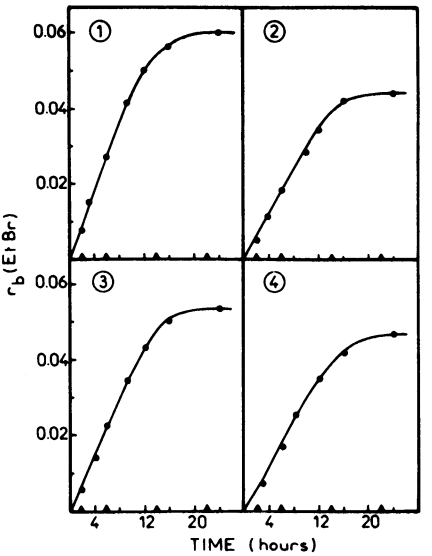

Figure 1 : Kinetics of binding of EtBr to double stranded polynucleotides. 1, poly(dG-dC).poly(dG-dC) ; 2, poly(dG).poly(dC) ; 3, poly(dA-dC).poly(dG-dT) ; 4, M. luteus DNA. The reaction mixtures were incubated at 37°C in 5 mM $NaClO_4$/1 mM phosphate buffer, pH 7.5. The input molar ratios cis-DDP and EtBr per nucleotide were respectively 0.08 and 0.25. (▲) are relative to experiments carried out in the same conditions but without cis-DDP. The amount of tightly bound EtBr per nucleotide, r_b, was determined by filter assay.

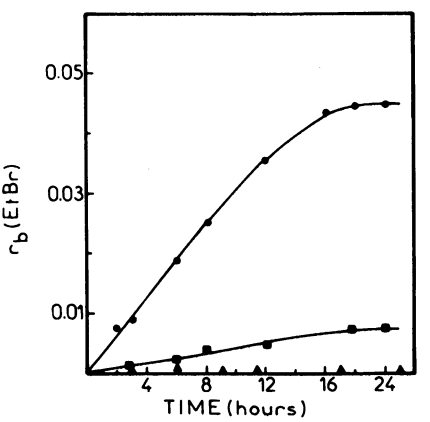

Figure 2 : Kinetics of binding of EtBr to nucleic acids. (●) native M. luteus DNA, (■) denatured M. luteus DNA, (▲) single stranded M 13 DNA. Same experimental conditions as in fig 1.

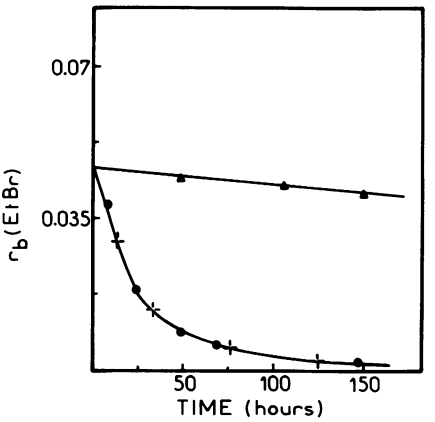

Figure 3 : Kinetics of release of tightly bound EtBr from the ternary complex M. Luteus DNA-Pt-EtBr. Experiments were carried out at pH 7.5 and at 4°C (▲) or 37°C (●). (+) are relative to experiments carried out at pH 9 or 11 and at 37°C. The solutions were extracted at various times with cold butanol. The values of r_b were determined by filter assay.

with native DNA and is negligible with single stranded M13 DNA. In the absence of nucleic acids, no reaction between cis-DDP and EtBr has been observed.

Stability of the ternary complexes

After incubation of DNA, EtBr and cis-DDP reaction mixture at 37°C during 24 hours, the solution was extracted with butanol and then dialyzed in the cold. The stability of the ternary complex DNA-Pt-EtBr has been studied as a function of temperature. At 0°C and neutral pH, the complex is stable over a long period of time (fig 3). At 37°C there is a slow release of EtBr. The kinetics of release are independent of pH (it has to be pointed out that from time to time the released dye was removed by extraction with butanol). The amount of bound platinum is constant (not shown). These results support those previously reported for the ternary complex poly(dG-dC)-Pt-EtBr, i.e., the ternary complexes nucleic acid-Pt-EtBr are stable at low temperature but are relatively unstable as the temperature is raised.

Spectroscopy studies

Absorption. It is well known that the visible absorption spectrum of EtBr intercalated between base pairs of DNA is red-shifted as compared to the spectrum of the free dye in water, the maxima being respectively at 530 and 480 nm (21). In 10 mM $NaClO_4$, the visible spectra of the ternary complexes poly(dG-dC)-Pt-EtBr and DNA-Pt-EtBr are the same and are only slightly red-

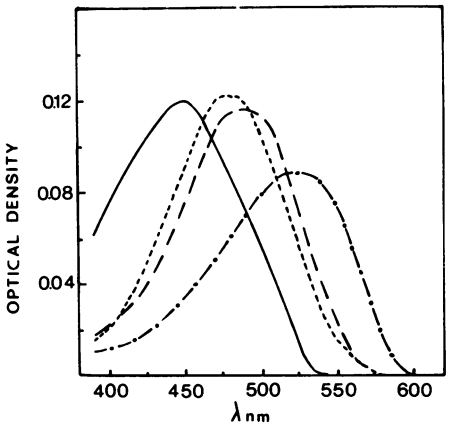

Figure 4 : Visible absorption spectra. (---) free EtBr in water ; (_ . _) EtBr in the presence of an excess of poly(dG-dC).poly(dG-dC) ; (_ _) ternary complex poly(dG-dC)-Pt-EtBr. In the three cases, the solvent is 10 mM $NaClO_4$/1 mM phosphate buffer pH 7.5. (___) ternary complex poly(dG-dC)-Pt-EtBr in 4 M $NaClO_4$/1 mM phosphate buffer pH 7.5. In all the samples, about the same amount of EtBr is present ($1.1x10^{-5}$).

shifted as compared to the spectrum of the free dye (fig 4). In 4 M $NaClO_4$, the spectrum of DNA-EtBr is almost unchanged (not shown) while that of poly (dG-dC)-Pt-EtBr is dramatically shifted, the maximum being near 445 nm. This suggests different contacts between the tightly bound dye and the solvent in low and high salt conditions.

Circular dichroism. The conformation of poly(dG-dC).poly(dG-dC) depends on salt conditions. The salt induced B form-Z form transition can be followed by circular dichroism, the spectrum of the Z form being almost an inversion of the spectrum of the B form (28). The circular dichroism spectra of poly (dG-dC).poly(dG-dC) in the absence and in the presence of EtBr and of poly(dG-dC)-Pt-EtBr in 100 mM and 4 M $NaClO_4$, respectively have been compared (fig 5).

In 100 mM $NaClO_4$ the spectrum of poly(dG-dC).poly(dG-dC) is slightly modified by the presence of the dye (the input molar ratio nucleotide residues over dye is equal to 0.06). The intensity of the first positive band is increased with a blue shift of the maximun (270 nm) while the intensity of the negative band centered at 250nm is almost unchanged. In the case of the ternary complex poly(dG-dC)-Pt-EtBr both the intensities of the positive band and of the negative band are more intense. In 4 M $NaClO_4$, the spectrum of poly(dG-dC).poly(dG-dC) is hardly modified by the presence of the dye. The

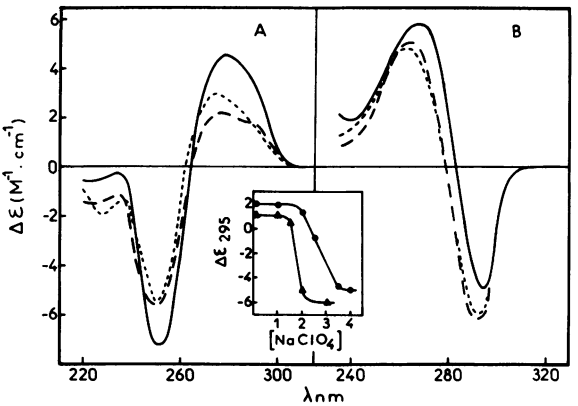

Figure 5 : Circular dichroism spectra in two salt conditions. A) 0.1 M $NaClO_4$/1 mM phosphate buffer pH 7.5. B) 4 M $NaClO_4$/1 mM phosphate buffer pH 7.5. (_ _) poly(dG-dC).poly(dG-dC), (---) poly(dG-dC).poly(dG-dC) plus EtBr, r_b = 0.06, (___) ternary complex poly(dG-dC)-Pt-EtBr, r_b = 0.06. Temperature 20°C. Inset : variation of $\Delta\varepsilon_{295}$ as a function of $NaClO_4$ concentration. (▲) poly(dG-dC).poly(dG-dC) or poly(dG-dC).poly(dG-dC), EtBr mixture, (●) ternary complex poly(dG-dC)-Pt-EtBr. After each addition of salt, the solutions were incubated 5 minutes at 40°C and 10 minutes at 20°C.

spectrum of poly(dG-dC)-Pt-EtBr is slightly different, with a first negative band, centered at 295 nm, less intense and a positive band, centered at 270 nm, more intense. In spite of these slight differences, the circular spectrum suggests that poly(dG-dC)-Pt-EtBr is in a Z-like conformation.

Three important points have to be underlined. The first one is that as judged by circular dichroism, the B form - Z form transition of the ternary complex poly(dG-dC)-Pt-EtBr is completely reversible. The second point is that the B form - Z form transition of the ternary complex occurs at higher salt concentration than that of poly(dG-dC).poly(dG-dC) in the presence or absence of EtBr, the midpoints of the transitions being respectively at 2.8 and 1.7 M $NaClO_4$ (inset fig 5). The third point is that the kinetics of B form-Z form transition of poly(dG-dC)-Pt-EtBr is much slower than that of poly (dG-dC).poly(dG-dC), EtBr mixture. In 2.8 M $NaClO_4$ and at 30°C the transition is complete after 3 and 12 minutes for poly(dG-dC).poly(dG-dC), EtBr mixture and poly(dG-dC)-Pt-EtBr, respectively. In 4 M $NaClO_4$ and at 30°C, the transition of poly(dG-dC)-Pt-EtBr is complete in about 5 minutes while that of poly(dG-dC).poly(dG-dC), EtBr mixture is too fast to be followed with our circular dichroism apparatus.

Fluorescence. The fluorescence quantum yield of EtBr intercalated between the base pairs of DNA is larger than that of free EtBr (21). The

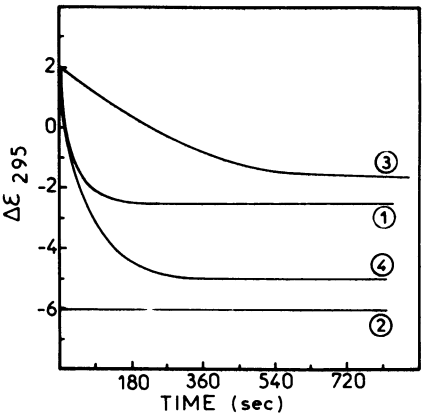

Figure 6 : Variation of $\Delta\varepsilon$ as a function of time. The salt jump was performed by addition of concentrated solutions of polynucleotides in 1 M $NaClO_4$ to a given salt solution, the final salt concentration being 2.8 M or 4 M $NaClO_4$, respectively. Curves 1 and 2 are relative to poly(dG-dC).poly(dG-dC) EtBr mixture (r_b = 0.06) in 2.8 and 4 M $NaClO_4$. Curves 3 and 4 are relative to the ternary complex poly(dG-dC)-Pt-EtBr (r_b = 0.06) in 2.8 and 4 M $NaClO_4$. The solutions contain 1 mM phosphate buffer pH 7.5. Temperature 30°C.

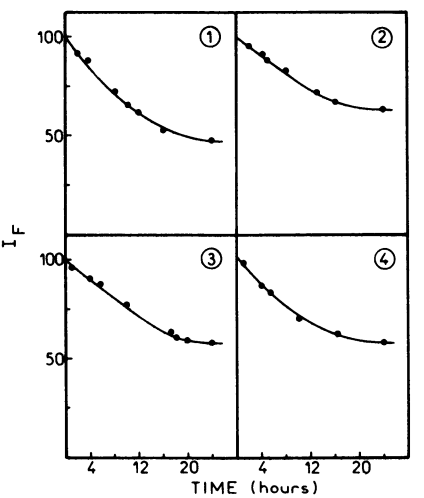

Figure 7 : Kinetics of binding of EtBr to double stranded polynucleotides followed by fluorescence. The fluorescence intensities (in arbitrary units) are plotted as a function of time ; λ_{exc} = 510 nm, λ_{em} = 590 nm. 1, poly(dG-dC).poly(dG-dC) ; 2, poly(dG).poly(dC) ; 3, poly(dA-dC).poly(dG-dT) ; 4, M. luteus DNA. Same experimental conditions as in fig 1.

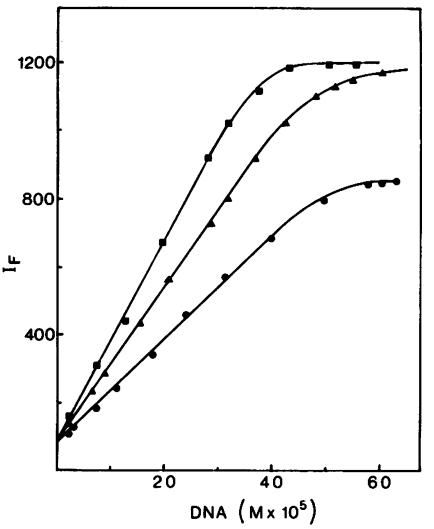

Figure 8 : Fluorescence of EtBr in the presence of DNA, platinated DNA and the ternary complex M. Luteus DNA-Pt-EtBr. To a solution of EtBr (6.8×10^{-5} M) were added increasing amounts of DNA (■), platinated DNA (▲) and DNA-Pt-EtBr (●). The platinated DNA and the ternary complex contain about the same amount of platinum ($r_b \simeq 0.08$) and the ternary complex contains tightly bound EtBr at $r_b \simeq 0.045$. Solvent 10mM $NaClO_4$/1mM phosphate buffer pH 7.5. λ_{exc} = 510 nm, λ_{em} = 590 nm. Temperature 18°C.

ternary complexes poly(dG-dC)-Pt-EtBr or DNA-Pt-EtBr are not fluorescent, i.e., their fluorescence is much weaker than that of free EtBr in water (not shown). These differences in the fluorescence quantum yields of EtBr respectively free, intercalated in DNA and tightly bound can be used to study the formation of the ternary complexes. As shown in fig 7, the fluorescence intensity of nucleic acid, cis-DDP and EtBr reaction mixtures decreases as a function of time and then remains constant. However, a quantitative analysis is difficult because the quantum yields of EtBr bound to a nucleic acid or to a ternary complex are different. This is illustrated in fig 8. To a given amount of EtBr were added increasing amounts of DNA, platinated DNA and DNA-Pt-EtBr, respectively. The fluorescence intensities increase and then remain constant. More platinated than unplatinated DNA is necessary to reach the plateau but at the plateau, both intensities are the same. On the other hand, in the presence of DNA-Pt-EtBr, the intensity at the plateau is smaller. A systematic determination of the quantum yields of EtBr bound to various ternary complexes is in progress in the laboratory.

Binding spectrum of platinum adducts

Numerous studies have shown that cis-DDP binds preferentially to $(dG)_n.(dC)_n$ sequences (3,14,22-25). However, the presence of some intercalating compounds interferes with this preferential binding (14-17). In order to better characterize the importance of tightly bound EtBr, we have examined the BamH1/Sal1 generated 275-bp restriction fragment from pBR322 modified by cis-DDP in the presence or absence of EtBr by means of the 3' →5' exonuclease activity of T4 DNA polymerase (19). This enzyme can completely digest this fragment of DNA. Analysis of the digest by gel electrophoresis does not reveal any stopping site (results not shown and ref 19). On the other hand, after covalent binding of bulky substituents such as acetylaminofluorene the enzyme is stopped near the modified nucleotides which allows the determination of the binding spectra of these bulky substituents (19,26,27). In fig 9 are shown the sequencing gels of the 275-bp fragments modified by cis-DDP along with the corresponding Maxam-Gilbert sequence ladders. Because of the gel resolution, only the bands between positions 520 and 620 are analysed. In a first approximation, the same pattern is found for the modified fragments at r_b equal to 0.002 and 0.005, respectively (lanes 1 and 2). Comparison of lane 3 (the platinated fragment, r_b = 0.005, has been incubated in 0.2 M NaCN for 12 hours at 37°C) and the chemical cleavage patterns obtained for adenine and guanine residues indicates that cis-DDP binds to d(GpG) and to d(ApG) doublets. It does not bind to d(GpA) doublets. There is a constant shift of about 2.5 nucleotides towards the higher molecular weights in the platinated fragments (lanes 1 and 2) as compared to the chemically generated guanine cleavage fragments. This shift is reduced to 0.5 nucleotide after removal of the platinum (lane 3).

The 2.5 nucleotides shift can be explained in the following way : 1) one nucleotide shift : the enzyme cleaves the phosphodiester bond in platinated d(GpG) and d(ApG) but the guanine residues are still cross-linked to the fragment. 2) 1.5 nucleotide shift : the presence of platinum slows down the migration by charge and mass effects (the charge effect is expected to increase as the size of the fragments decreases). After incubation with NaCN, one platinum residue and one (5')dGMP are removed and thus the fragments migrate slower than the corresponding Maxam-Gilbert (G) fragments which have a terminal 3'-phosphate.

Several comments have to be made. Incubation of platinated fragments with NaCN removes platinum completely from platinated d(ApG) on one hand and on the other hand incompletely from platinated d(GpG).

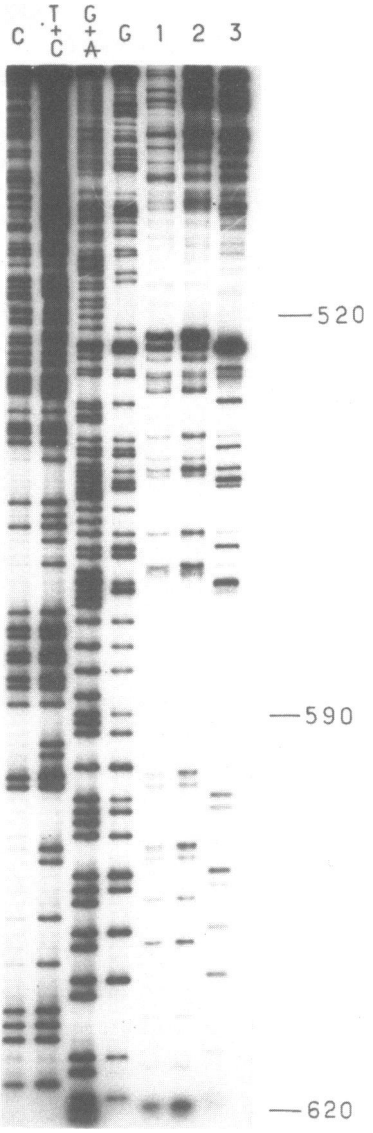

Figure 9 : Reactivity of the lower strand of pBR322 to cis-DDP. Analysis of the products obtained by digestion of platinated BamH1-Sal1 restriction fragment by means of the 3' → 5' exonuclease activity of T4 DNA polymerase on a 8 % sequencing gel. The BamH1(375)-Sal1(650) restriction fragment is (5'-^{32}P)-end-labelled at the Sal1 extremity. Lane 1, cis-DDP modified DNA fragment at a r_b = 0.002 ; lane 2, r_b = 0.005 ; lane 3, the sample r_b = 0.005 has been incubated in NaCN before the electrophoresis. The lanes labelled C, C+T, A+G and G are Maxam-Gilbert sequencing reactions of the same unplatinated restriction fragment.

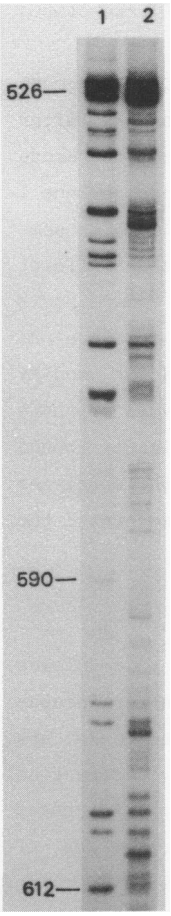

Figure 10 : Reactivity of the lower strand of pBR322 to cis-DDP in the presence of EtBr. Analysis of the products obtained of BamH1-Sal1 restriction fragments platinated in the absence or in the presence of EtBr by means of the 3'→ 5' exonuclease activity of T4 DNA polymerase on a 8 % sequencing gel. cis-DDP modified fragment (r_b = 0.005) in the absence of EtBr, lane 1, and in the presence of EtBr, lane 2.

All the d(GpG) doublets and all the d(ApG) doublets but one react with cis-DDP. There is no stop at the d(ApG) doublet (or a very faint stop) in the sequence d(GpApG) (position 590). This means that either this doublet does not react with cis-DDP or another adduct such as a cross-link between the two guanines is formed, an adduct which would not be a stop for the enzyme. In fact, this enzyme gives only a qualitative aspect of the cis-DDP binding spectrum. After incubation of a given amount of platinated restriction fragment with increasing amounts of enzyme, the sequencing patterns were the same but the intensities of the bands decreased (results not shown). The enzyme is slowed down by platinated d(ApG) and d(GpG) but is not completely stopped as in the case of bulky substituents. Thus, from these experiments, one cannot

deduce exactly the relative percentages of modification at d(ApG) and d(GpG) sites and one cannot be sure to detect all the adducts.

In fig 10 are shown the sequencing gels of the restriction fragment platinated in the absence (lane 1) or in the presence of EtBr (lane 2) (after the platination reaction, the solution was extracted with butanol to remove all the EtBr which was not tightly bound to DNA). Some of the stops in lane 1 are also present in lane 2. In addition there are new stops in lane 2, some of them being very intense as at the bottom of the gel. There are also faint stops near the d(GpApG) sequence and near the G surrounded by C (near position 581). It has been verified that small amounts of EtBr (as in lane 2) added to the unplatinated sample or to the platinated sample did not modify the sequencing patterns (results not shown). A quantitative analysis of these results is not yet possible because it is not known how the tightly bound EtBr stops the enzyme. Nevertheless, these results show that the complexes DNA-Pt-EtBr and platinated DNA plus EtBr behave differently and that the presence of EtBr modifies the binding spectrum of cis-DDP.

DISCUSSION

In this paper, we report some properties of the ternary complexes formed in the reaction between cis-DDP, EtBr and nucleic acids. After incubation of the reaction mixtures at 37°C for several hours, some EtBr are tightly bound to nucleic acids, i.e., they cannot be removed by extraction with butanol, by filtration assay at acid pH or by tlc at basic pH. As judged by these three assays, there is no tightly bound EtBr after incubation of EtBr and nucleic acids or of EtBr and platinated nucleic acids (the nucleic acids having been modified by cis-DDP and then added to the EtBr solution).

Some conditions are necessary to obtain these stable ternary complexes. All the added EtBr can be removed by any of the three assays if cis-DDP is replaced by trans-DDP. Intercalation of the dye between the base pairs of the nucleic acid is critical since there is no tightly bound EtBr with single stranded DNA. It is worth noting that in the absence of nucleic acid, cis-DDP does not react with EtBr (14,15,17). The sequence of the nucleic acid plays an important role. The molar ratio r_b (tightly bound EtBr per nucleotide) is larger with poly(dG-dC).poly(dG-dC) than with poly(dG).poly(dC) and more generally r_b[poly(dG-dC)] > r_b[poly(dA-dC).poly(dG-dT)] > r_b(DNA) > r_b[poly(dG).poly(dC)]. On the other hand in all these assays the amount of bound platinum is about the same and almost equal to the amount of added cis-DDP (this input ratio was always smaller than 0.1).

The stability of the ternary complexes (these complexes were obtained by incubation of cis-DDP, EtBr and nucleic acids reaction mixtures at 37°C for about 20 hours followed by several butanol extraction) depends upon temperature. At 0°C, the complexes are stable over a long period of time. At 37°C, there is a slow release of the tightly bound EtBr over a period of about 100 hours. The kinetics of release are pH-independent, in the range 7-11. On the other hand, no release of bound platinum has been detected.

This relative instability of the ternary complexes prevents any purification of the adducts after enzymatic digestion of the complexes. Nevertheless, the results previously reported on the ternary complex poly(dG-dC)-Pt-EtBr (17) and the spectroscopic study here described strongly suggest that in these complexes cis-DDP cross-links a guanine residue and an EtBr.

The fluorescence experiments support an interaction between EtBr and platinum or at least, a close contact in the ternary complexes. These complexes are not fluorescent, i.e., their fluorescence is even smaller than that of the corresponding amount of dye in water. The fluorescence quenching can be explained by the so-called "heavy atom effect", the dye and platinum being over distances of the order of van der Waal's radii (36).

In low salt conditions, visible spectra of the ternary complexes are similar, with a maximum near 485 nm. In high salt conditions, the spectrum of DNA-Pt-EtBr is almost unchanged but that of poly(dG-dC)-Pt-EtBr is blue-shifted with a maximum near 445 nm. As judged by circular dichroism, in low salt conditions the ternary complexes are in a B-like conformation and in high salt conditions, poly(dG-dC)-Pt-EtBr is in a Z-like conformation. Thus, the shift of the absorption spectrum of poly(dG-dC)-Pt-EtBr reveals a different environment of the dye. It seems reasonable to assume that in low salt conditions, EtBr is intercalated between the base pairs of the nucleic acids since the ternary complexes are not formed with single stranded DNA. This implies that the dye is more exposed to the solvent in the Z form than in the B form of poly(dG-dC)-Pt-EtBr. On the other hand, the visible spectrum of poly(dG-dC)-Pt-EtBr (Z form) is blue-shifted as compared to the spectrum of free EtBr in water. This can be explained by the binding of platinum to the dye.

A surprising result is that the fluorescence intensities of EtBr in the presence of excess of DNA or of DNA-Pt-EtBr respectively are not the same. It has been shown that platination of DNA decreases the number of EtBr binding sites (29). In agreement with this result, we find that more platinated DNA than unplatinated DNA is necessary to reach the same plateau value of the

fluorescence. On the other hand, a smaller plateau value is obtained by addition of DNA-Pt-EtBr. An explanation is that the tightly bound EtBr distorts the geometry of the double helix over several base pairs. These distorted sites can still bind EtBr but the fluorescence quantum yield of the dye intercalated in these sites is smaller than the quantum yield of the dye intercalated in the sites of unplatinated DNA.

It is known that EtBr inhibits salt induced transition of poly(dG-dC).poly(dG-dC) from the B to the Z form (30-33). The tightly bound EtBr stabilizes the B form of poly(dG-dC)-Pt-EtBr, the midpoint of the B form - Z form transition being at 2.8 M $NaClO_4$ for a sample in which the molar ratio tightly bound EtBr per nucleotide is equal to about 0.06. (The B form - Z form transition of this ternary complex is reversible). In the same experimental conditions, the midpoint of the B form - Z form transition of poly(dG-dC).poly(dG-dC) or of a mixture of this polynucleotide and EtBr (r_b = 0.06) is at 1.7 M $NaClO_4$. In fact, one has to compare the B form - Z form transitions of poly(dG-dC)-Pt-EtBr and of poly(dG-dC).poly(dG-dC) modified by a monofunctional platinum adduct since according to our hypothesis, in the ternary complex, one function of cis-DDP is bound to a guanine residue and the other to a dye. It has been shown that the modification of poly(dG-dC).poly(dG-dC) by the monofunctional platinum derivative chlorodiethylenetriaminoplatinum(II) is very efficient in stabilizing the Z form (34,35). Thus, there are two opposite effects in the ternary complexes, one (platinum residue) favoring the Z form and the other (tightly bound EtBr) strongly favoring the B form.

Previous results have shown that in the reaction of cis-DDP and DNA, EtBr interferes with the preferential binding of cis-DDP to $(dG)_n.(dC)_n$ sequences (14-17). The DNA binding spectra of cis-DDP in the presence or absence of EtBr have been determined by means of the exonuclease activity of T4 polymerase. One advantage of this enzyme over exonuclease III is that there is no sequence dependent slowing regions in unmodified DNA (19). First the BamH1/Sal1 generated 275 bp restriction fragment from platinated pBR322 has been examined. The sequencing gels show that the enzyme is stopped near all the (GpG) and (ApG) doublets and not near the (GpA) doublets. Assuming that band intensity on the gel corresponds to binding site frequency, the (GpG) doublets are more reactive than the (ApG) doublets which is in good agreement with complete enzymatic digestion studies (8,12). However, one (ApG) doublet in the sequence (GpApG) at position 590 is not platinated. It has to be pointed out that we do not know whether all the binding sites can be revealed

by this enzyme. In fact, the enzyme is slowed down but not completely stopped even at the platinated (GpG) and (ApG) sites since the platinated fragment can be entirely digested. A similar observation was made by D. Burnouf, M. Daune and R.P.P. Fuchs (private communication). Nevertheless, we think that by means of the 3'→5' exonuclease activity of T4 DNA polymerase one can get a qualitative determination of the major binding sites of cis-DDP to DNA. In addition, this study shows that incubation of platinated DNA with NaCN seems more efficient to remove platinum from platinated (ApG) sites than from platinated (GpG) sites.

Analysis of sequencing gels for the restriction fragments platinated in the presence of EtBr shows that the tightly bound dye introduces new stops for the enzyme. Some stops, very intense are located near (GpG) and (ApG) doublets ; some, less intense, are located near (G) which did not stop the enzyme in the fragment platinated without EtBr. It has been verified that the digestion patterns of the platinated or unplatinated fragments are not modified by addition of small amounts of EtBr. These preliminary results suggest that EtBr can modulate the binding of cis-DDP to DNA. However, one has to be cautious because it is not yet known with certainty how the tightly bound EtBr can disturb the enzymatic digestion of the fragments. A systematic study of several fragments is in progress in our laboratory.

At this point, we have to compare our results with those reported by Lippard and coworkers (14-16). By exonuclease III digestion of restriction fragments, they have shown that cis-DDP binds preferentially to oligo(dG) sequences and to a much lesser degree to (ApG) doublets. In addition they found that several sites containing (CpGpG) sequences are not modified. As already pointed out, in our fragment, all the (GpG) sites including the one in the (ApCpGpG) sequence at position 528 and all the (ApG) sites but one in the sequence (GpApG) (position 590) are platinated, even at very low level of platination (r_b = 0.003).

The comparison between the experiments on platinated DNA in the presence of EtBr is not straightforward because in their samples, the amount of tightly bound EtBr is not known. Nevertheless, in both studies, the enzymatic digestions reveal new platinum binding sites which gives some support to the modulation of cis-DDP binding to DNA by EtBr.

Our conclusion is that in the reaction of double stranded nucleic acids, cis-DDP and EtBr, the nucleic acid can be considered as a matrix achieving a favorable orientation of the reactants which makes possible the cross-linking by platinum of a dye and a base residue. Preliminary studies show that

proflavine but not acridine behaves like EtBr (17). Several intercalating agents are currently being assayed by in vivo and in vitro experiments in our laboratory.

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REFERENCES

1. Roberts, J.L. and Thomson, A.J. (1979) Prog. Nucleic Acid Res. Mol. Biol. 22, 71-133.
2. Prestayko, A.W., Crooke, S.T., and Carter, S.K., eds. (1980) Cisplatin : Current Status and Developments (Academic, New York).
3. Loehrer, P.J., and Einhorn, L.H. (1984) Anna. Intern. Med. 100, 704-713.
4. Pinto, A.L., and Lippard, S.J. (1985) Biochim. Biophys. Acta 780, 167-180.
5. Royer-Pokora, B., Gordon, L.K. and Haseltine, W.A. (1981) Nucleic Acids Res. 9, 4595-4609.
6. Tullius, T.D. and Lippard, S.J. (1981) J. Am. Chem. Soc. 103, 4620-4622.
7. Fichtinger-Schepman, A.M.J., Lohman, P.H.M. and Reedijk, J. (1982) Nucleic Acids Res. 10, 5345-5356.
8. Fichtinger-Schepman, A.M.J., van der Veer, J.L., den Hartog, J.H.J., Lohman, P.H.M. and Reedijk, J. (1985) Biochemistry 24, 707-713.
9. Johnson, N.P., Mazard, A.M., Escalier, J. and Macquet, J.P. (1985) J. Chem. Am. Soc. 107, 6376-6380.
10. Eastman, A. (1983) Biochemistry 22, 3927-3923.
11. Eastman, A. (1985) Biochemistry 24, 5027-5032.
12. Eastman, A. (1986) Biochemistry 25, 3912-3915.
13. Malinge, J.M. and Leng, M. (1984) Embo J. 3, 1273-1279.
14. Tullius, T.D. and Lippard, S.J. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 3489-3492.
15. Merkel, C.M. and Lippard, S.J. (1982) Cold Spring Harbor Symp. Quant. Biol. 47, 355-360.
16. Bowler, B.E. and Lippard, S.J. (1986) Biochemistry 25, 3031-3038.
17. Malinge, J.M. and Leng, M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6317-6321.
18. Malfoy, B., Hartmann, B., Macquet, J.P. and Leng, M.(1981) Cancer Res. 41, 4127-4131.
19. Fuchs, R.P.P. (1984) J. Mol. Biol. 177, 173-180.
20. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
21. Le Pecq, J.B. and Paoletti, C. (1967) J. Mol. Biol. 27, 87-106.
22. Stone, P.J., Kelman, A.D. and Sinex, F.M. (1974) Nature (London) 251, 736-737.
23. Stone, P.J., Kelman, A.D., Sinex, F.M., Bhargava, M.M. and Halvorson, H.O. (1976) J. Mol. Biol. 104, 793-801.
24. Cohen, G.L., Ledner, J.A., Bauer, W.R., Ushay, H.M., Caravana, C. and Lippard, S.J. (1980) J. Am. Chem. Soc. 102, 2487-2488.
25. Rahmouni, A., Malinge, J.M., Schwartz, A. and Leng, M. (1985) J. Biol. Struct. Dyn. 3, 363-375.
26. Granger-Schnarr, M., Daune, M.P. and Fuchs, R.P.P. (1986) J. Mol. Biol. 190, 499-507.

27. Koffel-Schwartz, N., Verdier, J.M., Bichara, M., Freund, A.M., Daune, M.P. and Fuchs, R.P.P. (1984) J. Mol. Biol. 177, 33-51.
28. Pohl, F.M. and Jovin, T.M.(1972) J. Mol. Biol. 67, 375-396.
29. Macquet, J.P. and Butour, J.L. (1978) Biochimie 60, 901-914.
30. Pohl, F.M., Jovin, T.M., Baehr, W. and Holbrook, J.J. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3805-3809.
31. Rio, P. and Leng, M. (1983) Nucleic Acids Res. 11, 4947-4956.
32. Mirau, P.A. and Kearns, D. (1983) Nucleic Acids Res. 11, 1931-1941.
33. Shafer, R.H., Brown, S.C., Delbarre, A. and Wade, D. (1984) Nucleic Acids Res. 12, 4679-4690.
34. Malfoy, B., Hartmann, B. and Leng, M. (1981) Nucleic Acids Res. 9, 5659-5669.
35. Ushay, H.M., Santella, R.M., Caradonna, J.P., Grunberger, D. and Lippard, S.J. (1982) Nucleic Acids Res. 10, 3573-3588.
36. Galley, W.C. and Purkey, R.M. (1972) Proc. Natl. Acad. Sci. USA 69, 2198-2202.